Quantitative and Qualitative Study of Intestinal Flora in Neonates

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ABSTRACT

Background: In the neonatal period the developing intestinal barrier function provides a sub-optimal mucosal defense against infection. Establishment of the normal commensal micro-flora plays a vital role in this process. **Aims:** To determine aerobic and anaerobic bacteria by quantitative and qualitative methods from faecal samples of neonates. **Settings and Design:** A prospective study was carried out in two groups in a tertiary care hospital, Group A-comprised preterm infant and in group B-full term infants. **Materials and Methods:** Sixty two preterm infants with the weight < 1500 gm and gestation age < 34 weeks and twenty nine full term infants with 4 weeks of age were included. Quantitation of bacterial load was done by ten-fold serial dilutions on respective media. **Statistical Analysis:** The data were analyzed by using EPIINFO-Ver 6.04. **Results and Conclusions:** The predominant aerobic bacterium was *Klebsiella pneumoniae*. In pre term infants aerobic bacteria were colonized with an average of 2.1 and anaerobic bacteria 0.1. Quantitation showed faecal bacterial colony count ranging from 10^4-10^{13} CFU/gms. Gram negative and gram positive bacteria increased gradually over an interval of 2 to 3 weeks. Mean log CFU of gram negative bacteria and gram positive bacteria were statistically insignificant from day 3 to day 14 ($P > 0.05$). On day 21 there was a significant change in colonization of both bacterial sp ($P < 0.05$). Potential pathogenic aerobic bacteria dominate the intestinal flora of premature babies nursed in neonatal unit. There is a need to investigate interventions to offset this imbalance in gut micro-ecology of premature babies.

Key words: Bacterial flora, Faecal, Intestine, Neonates, Quantitation

INTRODUCTION

In the neonatal period, establishment of normal commensal microflora plays an important role in the development of gastrointestinal mucosal defense. It has been reported that the bacterial flora of pre-term infants in neonatal intensive care unite (NICU) differs from normal term infants.[1,2] In the healthy, the gastrointestinal tract of term neonates were colonized by a variety of micro flora within 10 days of life, whereas in preterm infants microflora does not get an opportunity for colonization. In low birth weight infants, this is an important risk factor for neonatal mortality and morbidity, including sepsis and gastrointestinal disorders.[3,4] Most of these infants are usually treated with broad spectrum antibiotics under NICU conditions shortly after birth. So the bacterial flora of these infants are characterized by delayed colonization of gastrointestinal tract with limited number of bacteria.[5] However, there is limited literature available regarding the microbial flora in preterm infants especially in low birth weight babies (< 1500 g) who are predisposed to various infectious and gastrointestinal complication, such as Necrotizing Enterocolitis (NEC). Therefore this study was carried out with the objective of determining fecal microflora in premature infants admitted to NICU of a tertiary care hospital in order to evaluate the risk of developing gastrointestinal complication in these babies.

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MATERIALS AND METHODS

A prospective study (Phase-I) was carried out in two groups at All India Institute of Medical Sciences, New Delhi India after it was ethically cleared. In group A - sixty two pre term infants were enrolled with < 1500 g weight and gestation age were < 34 weeks. Of 62 cases, 34 stool samples were quantified and 28 were qualitatively analyzed for fecal bacterial flora. Samples were collected on day 3, 7, 14, 21, (all ± 2 days) in 34 babies. In group B - twenty nine full term babies were studied. Fecal samples were qualitatively analyzed.

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One gram of stool sample was transferred to a vial with glass beads (SIGMA Chemicals Co.USA), containing 1 ml of sterile normal saline and vortexed. A tenfold serial dilutions were prepared (10^2-10^9) and 10 µl of each dilution was cultured on to 5% sheep blood and MacConkey agar (Difco Chemicals) for aerobic bacteria. Plates were incubated at 37°C for 48 hours and examined after 24 hours and 48 hours. Bacteria were identified by using conventional biochemical tests as well as by API system i.e. API 20 E test, API staph/strep (bioMerieux Vitek Inc.), for various gram positive and negative organisms. For anaerobic culture, stool samples were inoculated on to Brain heart infusion agar (Hi-Media Laboratories, Pvt. Ltd. India) supplemented with haemin (Hi-Media Laboratories, Pvt. Ltd. India) and Vit K (Hi-Media Laboratories, Pvt. Ltd. India) and incubated anaerobically at 37°C for 48 hours to 72 hours. The anaerobic bacteria were identified by conventional biochemical methods and RapID ANA system (Innovative Diagnostic System Inc Norcross, GA).

The total numbers of bacteria per gram of stool was reported in each sample of quantitative study.

Statistics

Colony counts of organisms were expressed as $\log_{10}$ count. Mean log CFU count were used for statistical comparisons. The data were analyzed by using EPIINFO-Ver 6.04. The statistical technique for continuous variables were used by applying *t*-test or Mann-Whitney test (wherever applicable) and for categorical variables chi-square or Fisher exact test were used (to compare the number of infants colonized). Significance was seen if $P < 0.05$.

RESULTS

A total of 91 babies were included in this study to determine the intestinal flora pattern in neonates.

QUANTITATIVE ANALYSIS

Total colony counts of aerobic bacteria ranged between 10^4 to 10^{13} CFU/gm. Colonization rates for the most frequently isolated bacteria are summarized in Table 1. There was change in the colonization pattern at different intervals of time at the end of the sample collection (d21). Fecal bacteria both gram negative and gram positive increased gradually over an interval of 2 to 3 weeks. Some samples could not be collected at specific time interval because babies had not passed stool.

Colonization of gram negative organisms increased from day 1 to day 21, during the stay in the NICU. There was a consistent increase in the total number of bacterial species and significant changes in colonization pattern by individual species of bacteria over time. By the end of the first month of life a significant percentage of infant stool were colonized by *Klebsiella pneumoniae;* 17.8% at day 3, 50% at day 7, 61% at day 14, 60% at day 21 ($P<0.05$) and by *E. coli*; 35% at day 3, 40% at day 7, 38% at day 14 and 60% at day 21. Mean log CFU of gram negative bacteria and gram positive bacteria were statistically insignificant from day 3 to day 14 ($P > 0.05$). On day 21 there was significant change in colonization of both bacterial sp ($P < 0.05$) [Table 2]. *Salmonella senftenberg, Salmonella typhi, Salmonella gallinarum, Klebsiella oxytoca,* were recovered only in babies with clinical history of NEC.

QUALITATIVE ANALYSIS

The colonized gram negative and positive aerobic organisms were *Klebsiella pneumoniae* 41.3%, *Escherichia coli* 24.1%, *Proteus* sp 10.3%, *Enterococcus faecium* 34.4%, *Staphylococcus epidermidis* 6.8% and *Micrococcus* sp 3.4% [Table 3] and colonization of anaerobic organism were *Bifidobacterium* sp (13.4%) and *Clostridium bifermentans (*3.4%). There was statistically significant difference observed in the growth of gram positive organism in both groups ($P < 0.05$), which was found to be low in pre term infants. Growth of protective organism (beneficial bacteria) were statistically insignificant ($P > 0.05$) in both groups.

DISCUSSION

Bacterial colonization pattern in neonatal gut depends on various factors; gestational age, mode of delivery, type of feeding, bacterial interactions, antimicrobial therapy and other environmental factors. It has been reported that colonization of micro flora in breast fed differs from formula fed.[4,5] Some authors have found that fecal microflora of breast fed and formula fed infants are essentially the same; others have demonstrated that *Bifidobacteria* are the predominant flora in the faeces of breast fed infants[6,7] These studies have been focused on full term babies and suggested that gastrointestinal tract of healthy term neonates colonized with various types of aerobic and anaerobic microbial organisms.[8,9]

Iseki K studied the development of faecal flora, both aerobic and anaerobic on administering various formula feeds and observed the neonatal gut first colonized with members of *Enterobacteriaceae* on first day of life, which gradually decreases on 6th day followed by *Bifidobacteria*

Table 1: Total number of bacterial isolates in pre term infants (Group A)

Bacterial species	Quantitative study *n* = 34					Qualitative study *n* = 28	Total *n* = 62
	d3	d7	d14	d21	Total	d3	
	n = 27	*n* = 20	*n* = 18	*n* = 10	*n* = 34	*n* = 28	
Aerobic							
GNB							
K. pneumoniae	5	10	11	6	33	11	44
E. coli	10	8	7	6	31	9	40
S. senftenberg	0	1	1	0	2	0	2
S. typhi	0	1	0	0	1	0	1
S. gallinarum	1	0	0	0	1	0	1
P. aeruginosa	2	2	0	0	4	1	5
C. freundii	2	0	0	0	2	0	2
K. oxytoca	1	0	0	0	1	0	1
Pr. vulgaris	0	1	0	0	1	0	1
GPC							
E. faecium	2	2	1	1	6	3	9
Micrococcus sp.	1	1	0	0	2	0	2
S. epidermidis	0	1	1	1	3	0	3
S. aureus	1	1	0	0	2	0	2
Strep lactis	2	0	1	0	3	0	3
S. xylosus	1	1	0	0	2	0	2
E. faecalis	0	3	0	0	3	0	3
Aerococcus viridans	1	1	0	0	2	1	3
CONS	3	1	0	0	4	0	4
Anaerobic							
L. acidophillus	0	1	1	0	2	1	3
C. bifermentans	1	0	0	0	1	0	1
C. beijerinckii	0	0	1	0	1	0	1
Porphy. asaccharolytica	0	0	1	0	1	0	1
No sample	6	15	16	24	-	-	-

Table 2: Mean log CFU in babies < 1500 gm

Day	GNB (mean ± SD)	GPC (mean ± SD)	*P* value
3	7.27 ± 1.42	6.94 ± 1.61	0.55
7	7.08 ± 1.82	6.24 ± 2.09	0.25
14	6.82 ± 1.94	7.39 ± 1.37	0.58
21	7.60 ± 0.85	9.16 ± 1.39	0.04

Table 3: Total number of bacterial species in pre term and full term infants

Bacterial species	Qualitative study (Pre term infants) *n* = 28	Qualitative study (Full term infants) *n* = 29	Chi-square and Fisher exact test (*P* Value)
Aerobes (GNB)	21	22	0.76
Klebsiella pneumoniae	11 (39.2%)	12 (41.3%)	
E. coli	9 (32.1%)	7 (24.1%)	
Proteus sp	0	3 (10.3%)	
Pseudomonas aeruginosa	1	0	
Aerobes (GPC)	3	13	0.004
Enterococcus faecium	3 (10.7%)	10 (34.4%)	
Staphylococcus epidermidis	0	2 (6.8%)	
Micrococcus sp	0	1 (3.4 %)	
Staphylococcus xylosus	0	0	
Anaerobes	1	5	0.19
Bifidobacterium sp	0	4 (13.7%)	
Lactobacillus acidophillus	1 (3.5%)	0	
Clostridium bifermentans	0	1 (3.4%)	

that increased after 2-3 days of life breast fed infants. However, the numbers of other bacteria (enterobacteria) were more in the bottle fed than in breast fed infants. These results suggested that breast feeding is protecting the immature gut against systemic infection.[10] Ira HG *et al.* reported that population of gram positive bacteria were higher than gram negative bacteria in low birth weight babies and bacterial colonization pattern were similar in both breast milk fed and formula milk fed infants in US settings. They isolated *Lactobacillus* sp. and *Bifidobacterium* sp. in only one breast fed infant.[11] In another study it has been observed that members of *Enterobacteriaceae*, *Enterococcus* sp. and *Staphylococcus* sp. were colonized during 7th day of life without antibiotic therapy in term new born. In a study conducted by Stevens D K and workers, 20 infants fed with stored formula milk and proprietary formula in the second week of life among hospitalized preterm infants, demonstrated different pattern of intestinal flora from normal term infants that

may lead to nosocomial sepsis and increased incidence of systemic and localized infections. The use of frozen breast milk to suppress coliform and other potentially pathogenic organism is not shown to be effective in these hospitalized preterm infants, especially those who have been treated with broad spectrum antibiotics.[12,13] The present study was planned to determine various aerobic and anaerobic fecal bacterial flora of preterm infants (< 1500 gm) under NICU conditions. In our study most of the babies were colonized with 2-3 bacterial species in both groups, the predominant species being *Klebsiella pneumoniae* and *E.coli*. The colonization rate of gram negative bacteria was high in both pre term and term infants, whereas gram positive bacteria were less in numbers in preterm infants. Bacterial colonization of preterm gut is likely to have a role in the pathogenesis and predisposition to gastrointestinal complications such as Necrotizing enterocolitis (NEC). Westra-Meijer *et al.* investigated the aerobic and anaerobic fecal flora in neonatal necrotizing enterocolitis, to establish a relation between colonization with a particular bacterial flora and the development of NEC and reported the isolation rate of gram positive cocci (GPC) was higher than gram negative bacilli (GNB). Commonly isolated aerobic bacteria were *Staphylococcus epidermidis* (61%), *Enterococcus* sp. (56%), *Klebsiella* sp. (37%), and *E.coli* (29%) and anaerobic organisms were *Clostridium* sp (61%) and *Bacteroides* sp (20%). In their study, aerobic and anaerobic bacterial species colonized by an average of 2.1 and 0.8 per gram of stool respectively. They suggested that there was no significant difference in the colonization pattern of bacteria between study and control group. An important difference was that colonization of *Klebsiella* sp was higher in those babies who were diagnosed with the history of NEC,[14] whereas in current study *Salmonella senftenberg, E.coli, Salmonella typhi, Salmonella gallinarum,* and *Klebsiella oxytoca* were grown in those infants which were diagnosed with NEC. It has been suggested that pathogenic gut flora may play role as in predisposing neonates to nosocomial sepsis and NEC.[15,16] Hoy *et al.* reported that decline in the numbers of some bacterial species in the fecal flora due to change in pH after bacterial fermentation in small intestine, changes the intra-luminal conditions and precedes the clinical onset of NEC.[17]

Hall *et al.* found a deficiency of *Lactobacilli* as compared to coli-form bacteria in preterm infants who were nursed in NICU and a high count of *Lactobacillus* in full term infants by 30 days of age. This study indicates that there is a paucity of beneficial flora in preterm infants.[18] Aerobic and anaerobic bacterial species in preterm infants with or without NEC were colonized by an average of 2.1 and 0.1 per gram of stool respectively. In the present study *Lactobacillus* sp. was isolated in only one stool sample at 7-14 day, whereas in term babies *Bifidobacterium* sp were isolated in 4/29 (13.7%), this contrast with Rubatelli *et al.* from Italy who has reported a high prevalence of *Bifidobacterium* sp. (47.6%) in breast fed babies and Klessen *et al.* who reported in 89% of the breast fed babies on 7th day of life which increased to 95% by 30th day, *Lactobacilli* were isolated in 12/19 (63%) of stool samples at 7th day and increased to 80-85% over a period of two months.[19,20] However, it is surprising that in the present study *Lactobacilli* were not recovered from any stool sample of breast fed babies over a period of 1-2 months. Also the isolation of *Bifidobacterium* sp. in term babies was low as compared to the above study. In the wake of our finding, an important question to be considered is whether breast fed babies are better colonized with anaerobes in our country? Most of the preterm babies in NICU were receiving broad spectrum antibiotic therapy and fed with expressed breast milk partially or wholly, which may be a factor to limit bacterial colonization in the gut. There is a need to explore intervention that "normalizes" the gut microbial ecology. In healthy term babies breast milk promotes colonization of *Bifidobacterium* and *Lactobacillus* sp.[21] The indication for breast feeding merely on the grounds of helping normal intestinal flora development, to combat intestinal infection needs to be re-evaluated. The circulating environmental microbial pressure in developing country like India may be hindering colonization with anaerobic non-spore forming gram positive bacilli such as *Bifidobacterium* as well as *Lactobacillus*. In our previous study we have found a moderate degree of colonization of *Lactobacilli* in larger premature babies (> 1500gms) fed with a probiotic *Lactobacillus rahmnosus GG*. In recent years several studies including our study have shown that many *Lactobacillus* preparations can colonize the gut of pre mature neonates and modify the gut flora, but whether such a colonization results in clinically significant gain, still remain to be explored.[22,23]

The data presented here is based on microbiological isolation in conventional aerobic and anaerobic media. Recent reports suggests molecular tools based on 16 S rDNA sequence similarities such as fluorescent *in-situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) have helped to overcome limitations of conventional microbiological culture methods in studying the faecal microflora composition. These tools have been successfully applied to study the development of the infant microflora, changes in the human micro flora during aging, the effects of pre and probiotics on the human microflora composition, and the effects of dietary interventions on the intestinal micro flora.[24]

CONCLUSION

We conclude that specific microbial ecology of colonization pattern and lack of protective gram positive anaerobic bacteria especially *Bifidobacterium* sp. and *Lactobacillus* sp in immature gut, may be critical in the pathogenesis of NEC. It is speculated that the use of probiotics (*Lactobacillus* containing preparation) may have a protective role in these infants in order to prevent NEC and other gastrointestinal complications.

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